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02/15/02 JC784 U.S. PTO

PATENT APPLICATION TRANSMITTAL LETTER
(Small Entity)

Docket No. 01MEL1

TO THE ASSISTANT COMMISSIONER FOR PATENTS

J1040 U.S. PTO 10/077699 02/15/02

Transmitted herewith for filing under 35 U.S.C. 111 and 37 C.F.R. 1.53 is the patent application of:

Thomas Happe

For: **Hydrogen Production**

Enclosed are:

- ☒ Certificate of Mailing with Express Mail Mailing Label No. **EL 823589701 US**
- ☒ 5 sheets of drawings.
- ☒ A certified copy of a **prior provisional** application.
- ☒ Declaration ☒ Signed. ☐ Unsigned.
- ☒ Power of Attorney
- ☐ Information Disclosure Statement
- ☐ Preliminary Amendment
- ☒ 2 Verified Statement(s) to Establish Small Entity Status Under 37 C.F.R. 1.9 and 1.27.
- ☒ Other: **3.5 inch floppy disc, Self Addressed Stamped Postcard**

CLAIMS AS FILED

For	#Filed	#Allowed	#Extra		Rate	Fee
Total Claims	20	- 20 =	0	x	$9.00	$0.00
Indep. Claims	7	- 3 =	4	x	$42.00	$168.00
Multiple Dependent Claims (check if applicable) ☐						$0.00
					BASIC FEE	$370.00
					TOTAL FILING FEE	$538.00

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- ☐ Charge the issue fee set in 37 C.F.R. 1.18 at the mailing of the Notice of Allowance, pursuant to 37 C.F.R. 1.311(b).

Dated: **February 15, 2002**

Signature

Michael G. Petit, Reg. No. 30,795

cc: **Melis Energy, Thomas Happe**

CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Thomas Happe**			Docket No. **01MEL1**
Serial No. **Not Available**	Filing Date **Concurrent Herewith**	Examiner **Not Available**	Group Art Unit **Not Available**

Invention: **Hydrogen Production**

I hereby certify that this **original, nonprovisional utility patent application**
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PROVISIONAL APPLICATION COVERSHEET

Request under 37CFR§1.53(b)(2)

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Docket No:	01MEL1P	Type a plus sign ("+") inside this box →	+

INVENTOR(S)/APPLICANT(S)

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J1040 U.S. PTO
10/07699
02/15/02

TITLE OF THE INVENTION

Hydrogen Production

CORRESPONDENCE ADDRESS

Michael G. Petit
P. O. Box 91929
Santa Barbara

STATE: California ZIP CODE: 93190-1929 COUNTRY: US

ENCLOSED APPLICATION PARTS

- ☒ Specification: 10 Pages
- ☒ Small Entity Statement
- ☒ Drawings: 2 Sheets
- ☒ Claims: 2 Sheets
- ☒ OTHER: (Specify) Attachment A, 3.5 inch floppy disc bearing sequence data, 12 sheets printed sequence data

METHOD OF PAYMENT

- ☒ A check or money order is enclosed to cover the Provisional filing fees
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The invention was made by an agency of the United States Government or under contract with an agency of the United States Government: ☒ No. ☐ Yes, the name of the US Gov't agency and the contract number are:

_____

_____

Respectfully submitted,

Date: February 16, 2001

Michael G. Petit

Registration No: 30,795

☐ Additional inventors are being named on separately numbered sheets attached hereto.

Docket No.: 01MEL1P PROVISIONAL PATENT

IN THE UNITED STATES
PATENT AND TRADEMARK OFFICE

Applicant(s): Thomas Happe)
Filed : Concurrently Herewith)

15 February, 2001
Santa Barbara, California

TRANSMITTAL LETTER AND REQUEST FOR
FILING A PROVISIONAL APPLICATION UNDER 37 CFR§1.53(b)(2)

Honorable Commissioner of Patents
and Trademarks
Washington, D.C., 20231

Dear Sir:

This letter, together with the enclosed disclosure and cover sheet is a request for filing a Provisional Application under 37 CFR §1.53(b)(2).

1. Small Entity Status:

The applicant is a small entity under 37CFR§1.28(a).

2. Fee Payment:

Enclosed is a check totaling $75.00 for the Provisional Filing Fee under 37 CFR§1.16(k).

3. Power of Attorney:

A Power of Attorney to Michael G. Petit, Reg. No. 30,795 is enclosed herewith. In accordance therewith, please address all future

correspondence to: Michael G. Petit
P. O. Box 91929
Santa Barbara, CA 93190-1929
Phone: (805) 563-6556
FAX: (805) 563-6615

CERTIFICATION UNDER 37 CFR§1.10

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DATED: February 16, 2001

SIGNED: _____
Michael G. Petit
Registration No: 30,795

ABSTRACT

The enzyme iron hydrogenase (HydA) has industrial applications for the production of hydrogen, specifically for catalyzing the reversible reduction of protons to molecular hydrogen. The present invention relates to the isolation of a nucleic acid sequence from the green algae *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca* (Chlorophyta) that encodes iron hydrogenase. The invention further discloses the nucleic acid, m-RNA and the protein sequences for HydA. The genes and gene products may be used in a photosynthetic process for hydrogen production which includes growing a microorganism containing the HydA gene in a culture medium under illuminated conditions sufficient to accumulate biomass and endogenous substrate; then, upon scaling of the bioreactor, allowing the culture to become anaerobic by consumption of an endogenous or exogenous substrate and to produce hydrogen gas in the light.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the isolation of a green alga (Chlorophyta) nucleic acid sequence that encodes an enzyme that catalyzes the transfer of electrons to protons for the production of molecular hydrogen, and more particularly to iron hydrogenase and genes encoding for iron hydrogenase in eukaryotic photosynthetic microorganisms.

2. Prior Art

Molecular hydrogen is currently being considered as the ideal candidate for supplementing or replacing fossil fuels and, thereby, offering a source of clean energy. Among the potential methods for producing hydrogen for commercial purposes, the photosynthetic production of hydrogen by eukaryotic organisms is a desirable way of generating a renewable hydrogen fuel from sunlight and water, as the latter are among nature's most plentiful resources.

The ability of green algae (Chlorophyta), such as *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca*, to produce hydrogen from water has been recognized for about 60 years. This reaction is catalyzed by a so-called reversible hydrogenase, an enzyme that is induced in the cells after exposure to a short period of anaerobiosis. However, the activity of the hydrogenase is rapidly lost, as soon as the light is turned on, because of immediate inactivation of the reversible hydrogenase by photosynthetically generated O_2.

Methods have been devised to circumvent the hydrogenase inactivation problem. US Pat. No. 4,532,210 discloses the biological production of hydrogen in an algal culture using an alternating light and dark cycle. The process comprises alternating a step for cultivating the alga in water under aerobic conditions in the presence of light to accumulate photosynthetic products (starch) in the alga and a step for cultivating the alga in water under microaerobic conditions in the dark to decompose the accumulated material by photosynthesis to evolve hydrogen. This method uses a nitrogen gas purge technique to remove oxygen from the culture.

US Pat. No. 4,442,211 discloses that the efficiency of a process for producing hydrogen, by subjecting algae suspended in an aqueous phase to illumination, is increased by culturing algae which have been bleached during a first period of irradiation in a culture medium in an aerobic atmosphere until it has regained color and then subjecting this algae to a second period of irradiation wherein hydrogen is produced at an enhanced rate. A reaction cell is used wherein light irradiates the culture in an environment which is substantially free of CO_2 and atmospheric O_2. This environment is maintained by passing an inert gas (e.g. helium) through the cell culture to remove all hydrogen and oxygen generated by the splitting of water molecules in the aqueous medium. Although continuous purging of H_2-producing cultures with inert gases has allowed for the sustained production of H_2, such purging is expensive and impractical for large-scale mass cultures of algae. In view of the foregoing, there remains a need for a microorganism that produces a hydrogenase enzyme suitable for use in a sustainable

process of photosynthetic hydrogen production. There is a further need to clone the gene and identify the amino acid sequence of this hydrogenase as these have commercial applications for the production of hydrogen gas.

SUMMARY

Accordingly, it is an object of the present invention to provide a gene encoding for hydrogenase in green algae (Chlorophyta) and to enumerate methods for using the gene product for the microbial photo-production of molecular hydrogen. Specifically, the invention provides isolated nucleic acid sequences encoding an iron-hydrogenase enzyme (HydA) that will catalyze the reduction of protons to form molecular hydrogen.

Another object of the present invention is to provide isolated nucleic acid sequences encoding a protein that catalyzes the reduction of protons to form molecular hydrogen comprising SEQ. ID. NO. 1. SEQ. ID. NO. 1 comprises a nucleic acid sequence that encodes the *Scenedesmus obliquus* HydA.

It is a further object of the present invention to provide isolated nucleic acid sequences encoding a protein that catalyzes the reduction of protons to form molecular hydrogen comprising SEQ. ID. NO. 2. SEQ. ID. NO. 2 comprises a nucleic acid sequence that encodes *Chlamydomonas reinhardtii* HydA.

It is yet a further object of the present invention to provide isolated nucleic acid sequences encoding a protein that catalyzes the reduction of protons to form molecular

hydrogen comprising SEQ. ID. NO. 3. SEQ. ID. NO. 3 comprises a nucleic acid sequence that encodes *Chlorella fusca* HydA.

A further object of the present invention is to provide fragments of the nucleic acid sequence comprising SEQ. ID. NO. 1, or SEQ. ID. NO. 2, or SEQ. ID. NO. 3, encoding iron hydrogenase, that code for products that maintain the biological activity necessary to catalyze the transfer of electrons to protons in a process for producing molecular hydrogen. Such fragments can be either recombinant or synthetic or a combination thereof.

A still further object of the invention is to provide amino acid sequences for HydA encoded by nucleic acid sequences identified hereinabove as SEQ. ID. NOS. 1-3.

The features of the invention believed to be novel are set forth with particularity in the appended claims. However the invention itself, both as to organization and method of operation, together with further objects and advantages thereof may be best be understood by reference to the following description taken in conjunction with the accompanying drawings in which:

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of *Scenedesmus obliquus* HydA genomic and cDNA structures.

Figure 2 is a comparison of the *Scenedesmus obliquus*-derived iron hydrogenase amino acid sequence with HydA sequences derived from other organisms.

Figure 3 is a schematic representation of the genomic DNA structure from the green algae *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca*. The coding region of *hydA* is indicated as a broad band with the transit peptide shown in yellow. The untranslated 5'and 3'sequences are indicated as thin lines. The mosaic structure of *hydA* is indicated by bright colored boxes (introns) and dark colored boxes (exons). Introns are also marked, beginning with the 5' end of the sequences, by Latin numerals.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The isolation, purification and biochemical and genetic characterization of a first novel iron hydrogenase from green algae (Taxonomy: Eukaryota; Viridiplantae; Chlorophyta; Chlorophyceae) is disclosed in a paper (Attachment A hereto) submitted by the present inventor on September 15, 2000 for publication in: "The Journal of Biological Chemistry." The content of Attachment A, without limitation, is hereby incorporated herein by reference thereto. Attachment A discloses a monomeric enzyme, iron hydrogenase (HydA), having a molecular mass of 44.5 kDa (exclusive of the transit peptide associated therewith) derived from *Scenedesmus obliquus*. The polypeptide derived from the cDNA sequence has a length of 448 amino acids and is the smallest hydrogenase described to date. The nucleic acid sequence coding HydA in *Scenedesmus obliquus* is set forth in SEQ. ID. NO. 1 appended hereto.

In addition to the unicellular green algae *Scenedesmus obliquus*, discussed above, other algae within the order of Chlorophyta (e.g. *Chlamydomonas reinhardtii* and

Chlorella fusca) contain a gene *(hydA)* coding for a novel iron-hydrogenase enzyme (HydA), which is similar to that of *Scenedesmus obliquus*. This gene, through its encoded enzyme, catalyzes the synthesis of molecular hydrogen from protons and high potential energy electrons, and releases significant amounts of hydrogen gas, which is a valuable and clean source of energy. As with *Scenedesmus obliquus*, the process entails the utilization of sunlight and the oxidation of water or organic substrate in photosynthesis to generate reduced ferredoxin, which is the carrier of the high potential energy electrons. For the first time, the isolation, sequencing and characterization of this novel *hydA* genomic DNA, cDNA, precursor and mature iron-hydrogenase of photosynthetic eukaryotes is reported which may be used for hydrogen gas production.

The transcription of the iron-hydrogenase is very rapidly induced during anaerobic adaptation of the green algae. Hydrogen photoproduction by the cells can be observed soon following this induction. The genomic, cDNA and polypeptide sequences of the above-mentioned three representative green algae are offered as examples of the properties of the gene and of the enzyme that it encodes. The genomic DNA, cDNA and polypeptide sequences from three representative green algae are presented herein and are regarded as exemplary of the potential application of the *hydA* gene from any and all Chlorophyta in the process of commercial hydrogen production:

Scenedesmus obliquus

Genomic DNA: 5,001 bp, SEQ. NO. 1 (disc attached hereto)

cDNA: 2,636 bp, SEQ. ID. NO. 7 (disc attached hereto)

Precursor protein: 448 amino acids, 44.5 kD, SEQ. NO. 4 (disc attached hereto)

Mature protein: RRR amino acids, RRR kD

Chlamydomonas reinhardtii

Genomic DNA: 5,200 bp, SEQ. ID. NO. 2 (disc attached)

cDNA: 2,399 bp, SEQ. ID. NO. 8 (disc attached)

Precursor protein: 497 amino acids, 53.1 kD, SEQ. ID. NO. 5 (disc attached)

Mature protein: 441 amino acids, RRR kD

Chlorella fusca

Genomic DNA: 3,290 bp, SEQ. ID. NO. 3 (disc attached)

cDNA: 2,427 bp, SEQ. ID. NO. 9 (disc attached)

Precursor protein: 436 amino acids, RRR kD, SEQ. ID. NO. 6 (disc attached)

Mature protein: RRR amino acids, RRR kD

A schematic representation of *S. obliquus hydA* genomic and cDNA structures is shown in Figure 1. A, the coding region of the *hydA* cDNA is illustrated as a *large arrow* with the transit peptide shown in *black*. The untranslated 59 and 39 sequences are marked as *lines*. The *arrows* below indicate the sequencing strategy; each *arrow* represents an independent sequence determination. *TSP*, transcription start point; *ATG*, start codon. B, the mosaic structure of *hydA* is indicated by *gray* (exons) and *white boxes* (introns).

Figure 2 provides a comparison of the deduced HydA protein sequence with other iron hydrogenases. The protein alignment was done by using the Vector NTI program (InforMax). White letters with black background indicate amino acids identical to the

HydA protein. Black letters with graybackground indicate conserved changes of the amino acids. *S. o.*, *S. obliquus* (Exhibit A); *M. e.*, *Megasphaera elsdenii* (13); *D. d.*, *D. desulfuricans* (17); *T. v.*, *Trichomonas vaginalis* (10); *C. p.*, *C. pasteurianum* (9); *T. m.*, *T. maritima* (12); *N. o.*, *N. ovalis* (11). The parenthetical references cited hereinabove are appended to Exhibit A hereto.

Figure 3 is a schematic representation of the genomic DNA structure from the green algae *Scenedesmus obliquus*, *Chlamydomonas reinhardtii* and *Chlorella fusca*. The coding region of *hydA* is indicated as a broad band with the transit peptide shown in yellow. The untranslated 5'and 3'sequences are indicated as thin lines. The mosaic structure of *hydA* is indicated by bright colored boxes (introns) and dark colored boxes (exons). Introns are also marked, beginning with the 5' end of the sequences, by Latin numerals.

The new class of iron-hydrogenases described herein has a C-terminal portion and active site region (H-cluster) similar to that reported in non-photosynthetic prokaryotes (e.g. *Chlostridium pasteurianum*). Cysteine residues and distinct other amino acids which are strictly conserved in the active site (H-cluster) of such non-photosynthetic prokaryotes are also conserved in the iron-hydrogenase of green algae. However, the N-terminal region of the green alga iron-hydrogenase is substantially different from that of HydA in non-photosynthetic prokaryotes, revealing novel and unobvious pathways of electron transport for photosynthetic hydrogen production in green algae, as detailed below.

Distinct iron-sulfur [Fe-S] centers, referred to as FS2, FS4C, FS4B and FS4A, are encountered in the N-terminal region of the iron-hydrogenase in non-photosynthetic prokaryotes, and are thought to be instrumental in the transport of high potential energy electrons from bacterial ferredoxins to the catalytic site of the H-cluster of the hydrogenase [Peters et al., SCIENCE, 1998]. These distinct FS2, FS4C, FS4B and FS4A [Fe-S] centers are missing from the HydA of green algae. A mature-protein folding-model of the green alga iron-hydrogenase and analysis of its structure [Florin et al. 2001] revealed a protein region of positive surface potential, evidenced by the presence of basic amino acids, which are uniquely localized within the C-terminal domain and, therefore, near the catalytic site of the H-cluster. On the other hand, a model of the structure of green alga ferredoxin, which is different from prokaryotic ferredoxins, revealed negatively charged amino acids near the [2Fe-2S] electron donor site of this molecule [Bes et al. 1999]. This structural analysis revealed that the [2Fe-2S] center of green algal ferredoxin and the H-cluster of the hydrogenase probably come into close proximity through electrostatic interactions. This molecular geometry reveals a novel mechanism consisting of a direct and efficient electron transfer between the [2Fe-2S] center ferredoxin and the H-cluster that catalyzes hydrogen synthesis in green algae [Florin et al. 2001]. Thus, the hydA gene of green algae encodes an iron-hydrogenase polypeptide with a novel structure, one that uniquely permits a direct coupling and efficient electron-transfer from a [2Fe-2S] photosynthetic ferredoxin to the active site of the H-cluster. In support of this conclusion, inhibitor experiments revealed that the PetF ferredoxin

functions as a natural electron donor in green algae, linking the iron-hydrogenase with the photosynthetic electron transport chain in the chloroplast of these unicellular organisms.

In summary, a process, operable in a culture comprising green algae, is described whereby transcription of algal HydA genomic DNA, followed by translation of the resulting mRNA, followed by targetting of the precursor protein and import of the polypeptide into the chloroplast, followed by the mature iron-hydrogenase folding and catalysis, leads to hydrogen (H_2) gas production. While particular embodiments of the present invention have been illustrated and described, it would be obvious to those skilled in the art that various other changes and modifications can be made without departing from the spirit and scope of the invention. It is therefore intended to cover in the appended claims all such changes and modifications that are within the scope of this invention.

What we claim is:

CLAIMS

1. An isolated nucleic acid sequence comprising SEQ. ID. NO. 1.
2. A hydrogenase having an amino acid sequence comprising SEQ. ID. NO. 4.
3. An isolated nucleic acid sequence comprising SEQ. ID. NO. 2.
4. A hydrogenase having an amino acid sequence comprising SEQ. ID. NO. 5.
5. An isolated nucleic acid sequence comprising SEQ. ID. NO. 3.
6. A hydrogenase having an amino acid sequence comprising SEQ. ID. NO. 6.
7. A cell comprising an isolated nucleic acid sequence encoding a protein comprising SEQ. ID. NO. 4.
8. A cell comprising an isolated nucleic acid sequence encoding a protein comprising SEQ. ID. NO. 5.
9. A cell comprising an isolated nucleic acid sequence encoding a protein comprising SEQ. ID. NO. 6.
10. The process whereby the *hydA* genomic or cDNA sequences from green algae are used directly or upon modification of their promoter region in order to over-express the precursor/mature protein for purposes of enhancing yields of hydrogen production.
11. The process whereby *hydA* genomic or cDNA from green algae is used to transform other organisms (eukaryotic or prokaryotic, unicellular or multicellular, photosynthetic or non-photosynthetic) for purposes of hydrogen production.
12. The process whereby of *hydA* genomic or cDNA from green algae is used in random or targeted mutagenesis to alleviate, partially or totally, the natural

oxygen sensitivity of the green alga iron-hydrogenase, thus permitting hydrogen production under aerobic conditions.

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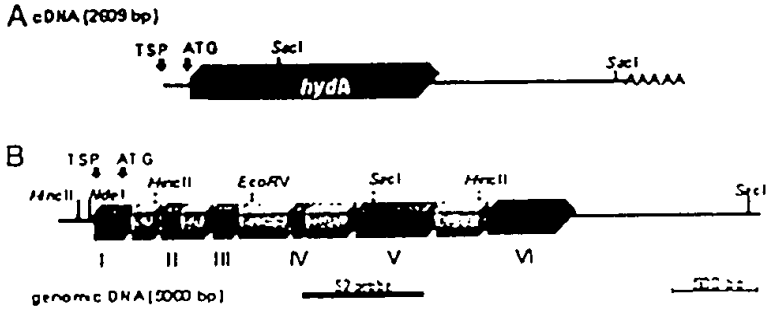


FIGURE 1

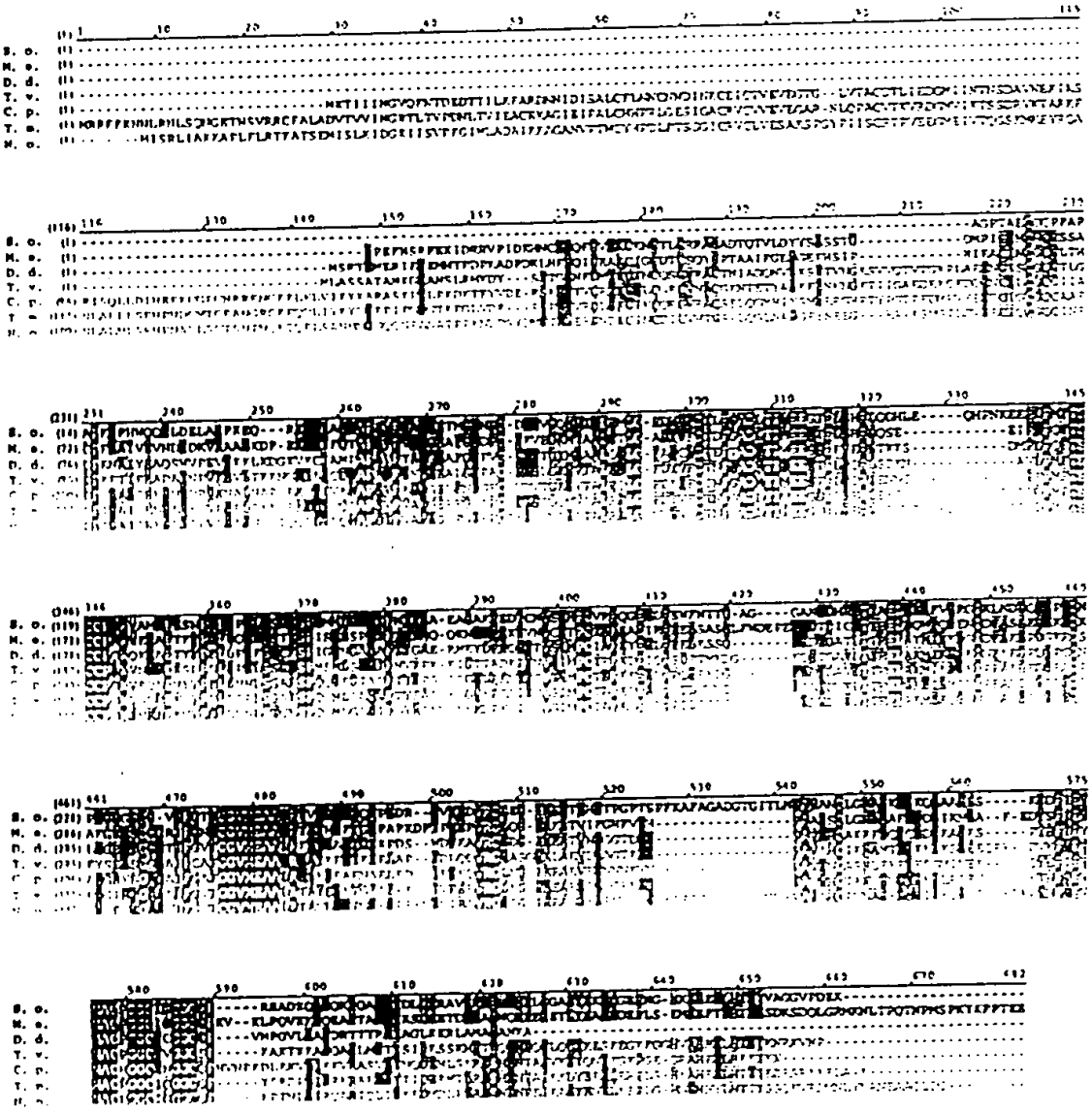
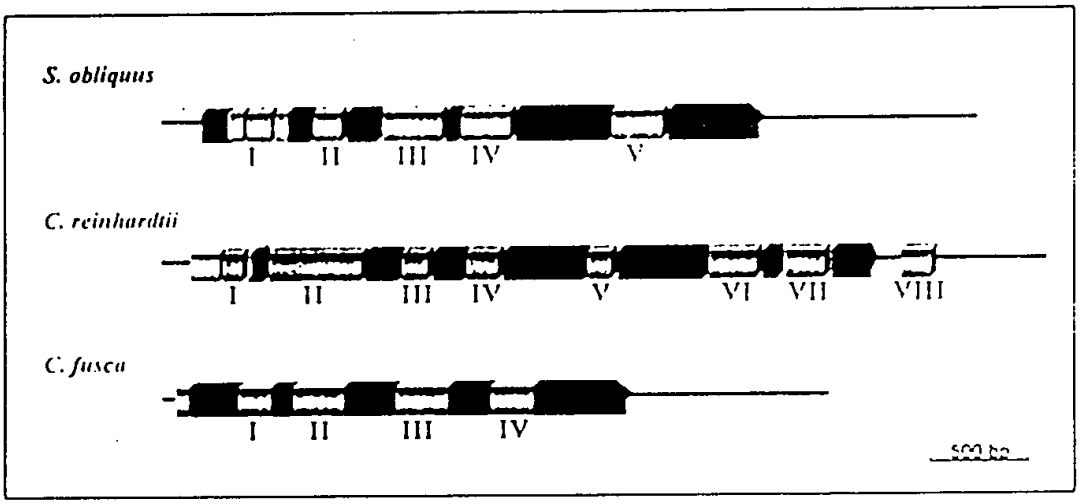


FIGURE 2


S. obliquus
I
II
III
IV
V
C. reinhardtii
I
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III
IV
V
VI
VII
VIII
C. fusca
I
II
III
IV
500 bp

FIGURE 3

ball3/bc-bc/bc-bc/bc0601/bc0455-01a spriggsd S=7 17/1/01 11:25 | Comments: | ARTNO: M008470200

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A Novel Type of Iron Hydrogenase in the Green Alga *Scenedesmus obliquus* Is Linked to the Photosynthetic Electron Transport Chain*

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Hydrogen evolution is observed in the green alga *Scenedesmus obliquus* after a phase of anaerobic adaptation. In this study we report the biochemical and genetical characterization of a new type of iron hydrogenase (HydA) in this photosynthetic organism. The monomeric enzyme has a molecular mass of 44.5 kDa. The complete *hydA* cDNA of 2609 base pairs comprises an open reading frame encoding a polypeptide of 448 amino acids. The protein contains a short transit peptide that routes the nucleus encoded hydrogenase to the chloroplast. Antibodies raised against the iron hydrogenase from *Chlamydomonas reinhardtii* react with both the isolated and in *Escherichia coli* overexpressed protein of *S. obliquus* as shown by Western blotting. By analyzing 5 kilobases of the genomic DNA, the transcription initiation site and five introns within *hydA* were revealed. Northern experiments suggest that *hydA* transcription is induced during anaerobic incubation. Alignments of *S. obliquus* HydA with known iron hydrogenases and sequencing of the N terminus of the purified protein confirm that HydA belongs to the class of iron hydrogenases. The C terminus of the enzyme including the catalytic site (H cluster) reveals a high degree of identity to iron hydrogenases. However, the lack of additional Fe-S clusters in the N-terminal domain indicates a novel pathway of electron transfer. Inhibitor experiments show that the ferredoxin PetF functions as natural electron donor linking the enzyme to the photosynthetic electron transport chain. PetF probably binds to the hydrogenase through electrostatic interactions.

Many prokaryotes and several eukaryotes have an enzyme complex in common catalyzing the reversible reduction of protons to molecular hydrogen. The diverse group of hydrogenases can be divided into three classes according to their metal composition in the active center (1). The nickel-iron hydrogenases are widespread among all bacteria families and have been well characterized during the last 30 years (2). The iron sulfur proteins consist of one to four subunits and have an additional nickel atom in the catalytic site (3, 4). In contrast, the iron hydrogenases possess only [Fe-S] clusters and an iron cofactor with an unique structure of six iron atoms (5, 6). The third class of hydrogenases lacks the iron sulfur clusters as well as additional metal atoms and was found only in methanogenic bacteria (7, 8).

Until now, iron hydrogenases have only been found in hydrogen-producing anaerobic bacteria and protozoa (9–13). The enzymes allow fermentative anaerobes to evolve H_2 without exogenous electron acceptors other than protons (14). They show a high specific activity that is about 100 fold higher compared with the nickel-iron hydrogenases (15). Furthermore, all iron hydrogenases are extremely sensitive to oxygen and carbon monoxide. The structures of the iron hydrogenases from *Clostridium pasteurianum* and *Desulfovibrio desulfuricans* have recently been investigated by x-ray crystallography (16, 17). The proteins consist of one or two subunits and have a remarkable iron cofactor (H cluster) in the catalytic site. The H cluster contains an unusual supercluster comprising a [4Fe4S] subcluster and a [2Fe] center, which are bridged together by a single cysteinyl sulfur (18). A number of conserved amino acids forms a hydrophobic pocket that shields the [2Fe] subcluster from the solvent. In all known iron hydrogenases at least eight conserved cysteines were found at the N-terminal site of the protein that coordinate two further [4Fe-4S] clusters (F cluster). It is discussed that the F clusters are responsible for the electron transfer from the surface of the protein to the active site (17, 19).

In green algae, Gaffron (20) discovered a hydrogen metabolism 60 years ago. After anaerobic adaptation, he observed both H_2 uptake and hydrogen evolution dependent on the CO_2 partial pressure (21, 22). After bubbling the cells with an inert gas like argon, high rates of H_2 production can be measured in the light (23). Electrons are supplied either by photochemical water splitting at photosystem II, which results in simultaneous production of hydrogen and oxygen, or by metabolic oxidation of organic compounds with release of CO_2 (24–27). Light-dependent electron transport from organic substrate through the plastoquinone pool to the hydrogenase provides the cells with ATP under anaerobic conditions (28, 29).

From the unicellular green alga *Chlamydomonas reinhardtii* a monomeric iron hydrogenase with high specific activity has been isolated (30, 31). In contrast, a nickel-iron hydrogenase was described for another well examined green alga, *Scenedesmus obliquus* (32, 33). The protein consists of two subunits of about 36 and 55 kDa and might be located in the chloroplast.

To investigate whether hydrogenases of the iron-only type also occur in green algae other than *C. reinhardtii*, we decided to look for the gene of a hydrogenase in *S. obliquus*. Interestingly, we isolated the protein and the gene encoding a monomeric iron hydrogenase (HydA). Although the H cluster of the HydA protein of *S. obliquus* is very conserved, the N-terminal site is completely different compared with other iron hydrogenases. Further cysteines are not present. These cysteine residues coordinate the typical F clusters that are necessary for the

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This paper is dedicated to Jan Vleek who found evidence for an iron hydrogenase in *S. obliquus* for the first time. Unfortunately, he died 4 years ago in a tragic car accident.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ271546.

‡ To whom correspondence should be addressed. Tel.: 49-228-732075; Fax: 49-228-731697; E-mail: t.happe@uni-bonn.de.

electron pathway in other iron hydrogenases. We performed physiological measurements of the hydrogen evolving activity in the present of chloroplast ferredoxin specific inhibitors as well as antibodies against this protein. The results clearly indicated that ferredoxin transfers electrons to the hydrogenase and links the enzyme to photosynthesis. The expression of the *hydA* gene is regulated at the transcriptional level. The mRNA is transcribed very rapidly during the process of anaerobic adaptation.

EXPERIMENTAL PROCEDURES

Algae Strains, Growth, and Anaerobic Conditions—Wild-type *S. obliquus* Kützing 276-6 was obtained originally from the culture collection of algae at the University of Gottingen. Cells were cultured photoheterotrophically (34) in batch cultures at 25 °C under continuous irradiance of 150 μmol photons × m^{-2} s^{-1}. For anaerobic adaptation, 4-liter cultures were bubbled vigorously with air supplemented with 5% CO_2. After harvesting the cells in the mid exponential stage of growth, the pellet was resuspended in fresh Tris acetate phosphate medium. The algae were anaerobically adapted by flushing the culture with argon in the dark.

Hydrogen Evolution Assay—The *in vitro* hydrogenase activity was measured by using a gas chromatograph from Hewlett Packard (HP 5890, Series II) equipped with a thermal conductivity detector and a molecular sieve column. Methylviologen reduced by sodium dithionite was used as electron donor as described before (30). 1 unit is defined as the amount of hydrogenase evolving 1 μmol H_2 × min^{-1} at 25 °C.

The *in vivo* activity in the presence of different inhibitors of the photosynthetic electron flow was determined as described (30). After anaerobic adaptation, cells were harvested, diluted in fresh Tris acetate phosphate medium, and transferred to sealed tubes. Inhibitors were added 1 h before H_2 evolving activity was measured. Cells were broken by sonification. Thylakoid membranes and photosynthetic electron transport chain remained intact as shown by O_2 polarography. Ferredoxin of *C. reinhardtii* and *S. obliquus* was isolated according to the method of Schmitter *et al.* (35).

Rapid Amplification of cDNA Ends-Polymerase Chain Reaction—RACE-PCR (36)[1] was performed with the SMART™RACE cDNA Amplification Kit (CLONTECH Laboratories, Palo Alto, CA) according to the manufacturer's recommendations except for modifications of the PCR and hybridization conditions. Starting material consisted of 1 μg of mRNA from anaerobically adapted cells. The reverse transcription reaction was carried out with a Moloney murine leukemia virus reverse transcriptase in two separate reaction tubes containing either the 5'- or the 3'-RACE-PCR specific primer from the kit. The cDNA of each sample served as template for the following PCR. For the 5'-RACE-PCR, Universal Primer Mix and the antisense primer Sc7 were used. The amplification of the 3'-cDNA end was performed with Universal Primer Mix and the sense primer Sc6. To obtain more distinct PCR signals, the PCR was repeated for both reactions with nested universal primers and designed primers (inverse Sc6 and inverse Sc7, respectively) using a dilution of the products of the first PCR as template.

Primer Extension—RACE-PCR was also implemented to map the transcription initiation site of the *hydA* mRNA (37). A gene-specific primer (Sc17) was used to carry out the first strand cDNA synthesis with the Supercript II reverse transcriptase (Life Technologies, Inc.) and 200 ng of mRNA as template. PCR was performed using either Sc12 or Sc27 and the SMART™ specific adapter primer Universal Primer Mix. Two different DNA fragments of 234 and 183 bp were amplified under standard PCR conditions. Both fragments were cloned into the pGem®T-Easy vector (Promega, Madison, WI) and sequenced using primers from the polylinker of the vector.

Genome Walking with Genomic DNA—Applying the CLONTECH Genome Walker Kit, genomic libraries from *S. obliquus* were generated by digestion with different blunt end cutting endonucleases (*NaeI*, *DraI*, *PvuII*, *HincII*, and *EcoRV*) and by adapter ligation at the ends of the resulting DNA fragments. These libraries were utilized as independent templates in five different PCR reactions (38). Two gene-specific primers (Sc27 and Sc35) derived from the *hydA* cDNA sequence of *S. obliquus* were used in combination with a kit adapter primer (AP1) in a first PCR reaction. Subsequently, 1 μl of the first PCR served as a template in a secondary PCR, applying two nested gene-specific primers (i-Sc10 and Sc32) along with a nested kit adapter primer (AP2). The resulting products were cloned into pGem®T-Easy and sequenced. Sequencing was performed by the dideoxy chain termination method (39).

Purification of the Iron Hydrogenase—40-liter cultures of *S. obliquus* were grown heterotrophically. After centrifugation (10 min at 5000 × *g*), the pellet was resuspended in 200 ml of Tris acetate phosphate medium. The cells were anaerobically adapted by flushing the solution with argon for 1 h in the dark. All further purification steps were performed in an anaerobic chamber (Coylab, town, MI). The cells were disrupted in a 50 mM Tris/HCl buffer, pH 8.0, 10 mM sodium dithionite by vortexing 3 min with glass beads. The further purification steps were made as described earlier for the isolation of the iron hydrogenase of *C. reinhardtii* (30). Automated Edman degradation of the N-terminal site of the protein was performed with an Applied Biosystem model 477A sequencer with online Analysator model 120 A.

RNA Blot Hybridization—Total RNA of *S. obliquus* was isolated according to the method described earlier (40). Equal amounts (20 μg) were separated electrophoretically on 1.2% agarose gels containing formaldehyde (41). The RNA was transferred onto nylon membranes (Hybond⁺, Amersham Pharmacia Biotech) and hybridized with RNA probes labeled with DIG-dUTP using the *in vitro* transcription method. A 1.3-kilobase *Eco*RI cDNA fragment was used to detect transcripts of the *hydA* gene, whereas a DIG-dUTP-labeled cDNA encoding constitutively expressed plastocyanin (42) was used as control. Hybridization reactions were carried out using protocols supplied by the manufacturer (Roche Molecular Biochemicals).

Sequence Analysis Software—Nucleic acid and protein sequences were analyzed with the programs Sci Ed Central (Scientific Educational Software) and ClustalW (43). The Blast server (44) of the National Center for Biotechnology Information (Bethseda, MD) was used for data base searches.

Recombinant Expression in Escherichia coli—The *hydA* open reading frame was amplified by PCR using the primer pair Sc29 and Sc30 containing flanking *NdeI*-*BamHI* sites. The PCR product was cloned into the pGem®T-Easy vector. After digestion with *NdeI*-*BamHI*, the *hydA* gene was cloned into the corresponding site of the pET9a expression vector (Promega) producing pLF29.2. The insert of pLF29.2 was sequenced confirming that the fragment contains the exact full coding region of the hydrogenase without transit peptide. *E. coli* strain BL21(DE3)pLysS was transformed with pLF29.2. Expression was induced with 1 mM isopropyl-thio-β-D-galactoside at an A_{600} of 0.3. Pel-

TABLE I

Biochemical data comparison of purified iron hydrogenases from C. reinhardtii and S. obliquus

	C. reinhardtii	*S. obliquus*
Size	49 kDa	44.5 kDa
Specific activity	935 units/mg protein	700 units/mg protein
Temperature optimum	60 °C	60 °C
pH optimum	6.9	7.3
Localization	chloroplast stroma	chloroplast
Coding site	nuclear	nuclear
pI value	5.3	5.17
K_M value (MV)	830 μM	800 μM
K_M (ferredoxin)	35 μM	ND*

* ND, not determined.

TABLE II

Effects of different photosynthetical inhibitors on hydrogenase activity

After anaerobic adaptation, cells were harvested, diluted in fresh TAP medium, and incubated with inhibitors as described under "Experimental Procedures." α-PetF-antibody was raised against spinach ferredoxin. DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyle-*p*-benzochinone; Sulfo-DSPD, sulfo-disalicylidinepropanediamin; DCPIP, 2,6-dichlorophenolindophenol.

	Hydrogenase activity
	units/mg chlorophyll
Intact cells (control)	0.11
- DCMU (10^{-5} M)	0.10
- DBMIB (10^{-5} M)	0.005
Broken cells	0.1
- DCMU (10^{-5} M)	0.11
- DBMIB (10^{-5} M)	0.006
- DCPIP (10^{-4} M)	0.003
- sulfo-DSPD (10^{-4} M)	0.003
- α-PetF-antibody (1:1000)	0.005

[1] The abbreviations used are: RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis.

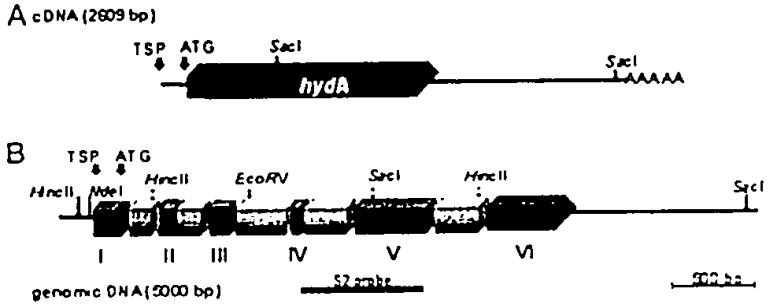

FIG. 1. **Schematic representation of *S. obliquus hydA* genomic and cDNA structures.** *A*, the coding region of the *hydA* cDNA is illustrated as a *large arrow* with the transit peptide shown in *black*. The untranslated 5' and 3' sequences are marked as *lines*. The *arrows* below indicate the sequencing strategy; each *arrow* represents an independent sequence determination. *TSP*, transcription start point; *ATG*, start codon. *B*, the mosaic structure of *hydA* is indicated by *gray* (exons) and *white boxes* (introns). The S2 probe and different restriction enzymes that were used in the Southern blot experiments are mentioned.

AQ: H

leted cells were resuspended in lysis buffer (100 mM Tris/HCl, 4 mM EDTA, 16% glycine, 2% SDS, 2% mercaptoethanol, 0.05% bromphenol blue, 8 M urea). After heating, the protein extract was separated by 10% SDS-PAGE and blotted onto a polyvinylidene difluoride membrane.

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Western blot analyses were performed using antisera against the iron hydrogenase of *C. reinhardtii* at 1:1000 dilution as described earlier (31).

RESULTS

Induction of Hydrogenase Activity and Purification of the Iron Hydrogenase Protein—Anaerobic adaptation is the most efficient way to induce hydrogenase activity in *S. obliquus*. Bubbling the alga culture in the dark with argon led to a dramatic increase (10-fold) of hydrogenase activity during the first 2 h. We purified the enzyme of *S. obliquus* to homogeneity by successive column chromatography. Because the enzyme is irreversible inactivated by lowest oxygen levels, all purification steps were performed under strictly anaerobic conditions and in the presence of reducing agents (dithionite). The purification scheme resulted in a 5200-fold purification of HydA with 5% recovery (data not shown). The most powerful step for purifying the protein was a Q-Sepharose high performance column chromatography with pH gradient elution. Gel filtration chromatography of hydrogenase on a calibrated Superdex-75 column resulted in a single activity peak corresponding to a molecular mass of 45 kDa. The monomeric structure of the enzyme could also be shown on a SDS-polyacrylamide gel after Coomassie Blue staining (data not shown). The N-terminal sequence of HydA was determined by Edman degradation. The protein sequence (AGPTAECDRPPAPAPKAAHWQ) is, except for two amino acids, identical to the amino acid sequence deduced from the DNA data (AGPTAECDCPPAPAPKAPHWQ). In the course of our purification procedure, we never found a hint for a second hydrogenase in *S. obliquus* because the hydrogenase activity was never separated in several distinct fractions. Biochemical data show a high similarity of HydA to the iron

T1

hydrogenase from *C. reinhardtii* (Table I). The enzymes have a high temperature optimum of about 60 °C, are strongly inhibited by O_2 and CO, and catalyze the H_2 evolution with a typical high specific activity. Experiments with inhibitors of translation on ribosomes (data not shown) and analysis of the gene structure (see below) show that HydA from *S. obliquus* is translated in the cytoplasm and then transported into the chloroplast.

Ferredoxin Is the Natural Electron Donor of the Iron Hydrogenase—Hydrogenase activity was determined in intact and broken cells after anaerobic adaptation. The integrity of the photosynthetic electron transport in the sonified cell preparation was demonstrated by the rate of oxygen evolution (154

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μmol O_2/mg Chl × h). This rate corresponds to 85% of the oxygen evolution measured with intact *Scenedesmus* cells.

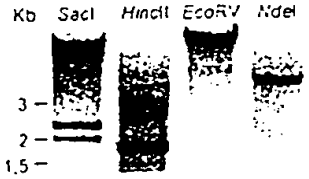

FIG. 2. **The *hydA* gene is located in the genome of *S. obliquus* as a single copy gene.** Southern analysis was carried out by digesting genomic DNA of *S. obliquus* with four different restriction endonucleases (*Sac*I, *Hin*cII, *Eco*RV, and *Nde*I). 10 μg of DNA was loaded per lane. The S2 DNA-Probe (750 bp) was used for the hybridization as indicated in Fig. 1.

In *S. obliquus*, the hydrogen evolution is linked to the photosynthetic electron transport chain through PSI. As shown in

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Table II, the cells were still able to photoproduce hydrogen when electron flow of PSII was blocked by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. In contrast, addition of 2,5-dibromo-3-methyl-6-isopropyle-*p*-benzochinone resulted in inhibition of the H_2 production, thus giving evidence of the involvement of PSI in the supply of electrons to hydrogenase. The electron transport from PSI to ferredoxin was inhibited using the artificial electron acceptor 2,6-dichlorophenolindophenol. In this reaction, 2,6-dichlorophenolindophenol is reduced instead of ferredoxin, and the electron transfer to hydrogenase is interrupted.

Hydrogenase activity was dramatically reduced (up to 30-fold) by the ferredoxin antagonist sulfo-disalicylidinepropandiamine (Table II). Similar results were achieved with α-PetF-antibodies that specifically recognize the ferredoxin protein. In both cases, the hydrogenase enzyme can not evolve hydrogen, thus demonstrating the role of ferredoxin as the obligatory electron donor for the hydrogenase reaction.

The electron transfer properties of different plant-type ferredoxins were measured *in vitro* with dithionite as reducing reagent. The ferredoxin proteins of spinach, *C. reinhardtii*, and *S. obliquus* were comparable regarding their capability to reduce purified *S. obliquus* hydrogenase. In this assay, we obtained H_2-evolving activities of 420, 390, and 350 units/mg protein with *S. obliquus*, *C. reinhardtii*, and spinach ferredoxin, respectively. No hydrogen production could be measured with other possible electron donors like cytochrome and NADPH. In *D. desulfuricans* the iron hydrogenase was reported to catalyze both hydrogen production and uptake with low potential multiheme cytochromes like cytochrome c_3 (17).

Molecular Characterization of hydA Encoding an Iron Hydrogenase—To isolate the gene encoding a iron hydrogenase in *S. obliquus*, we isolated poly(A)$^+$ RNA from cell cultures after 1 h of anaerobic adaptation. Isolated RNA was transcribed and amplified by reverse transcription-PCR using oligonucleotides

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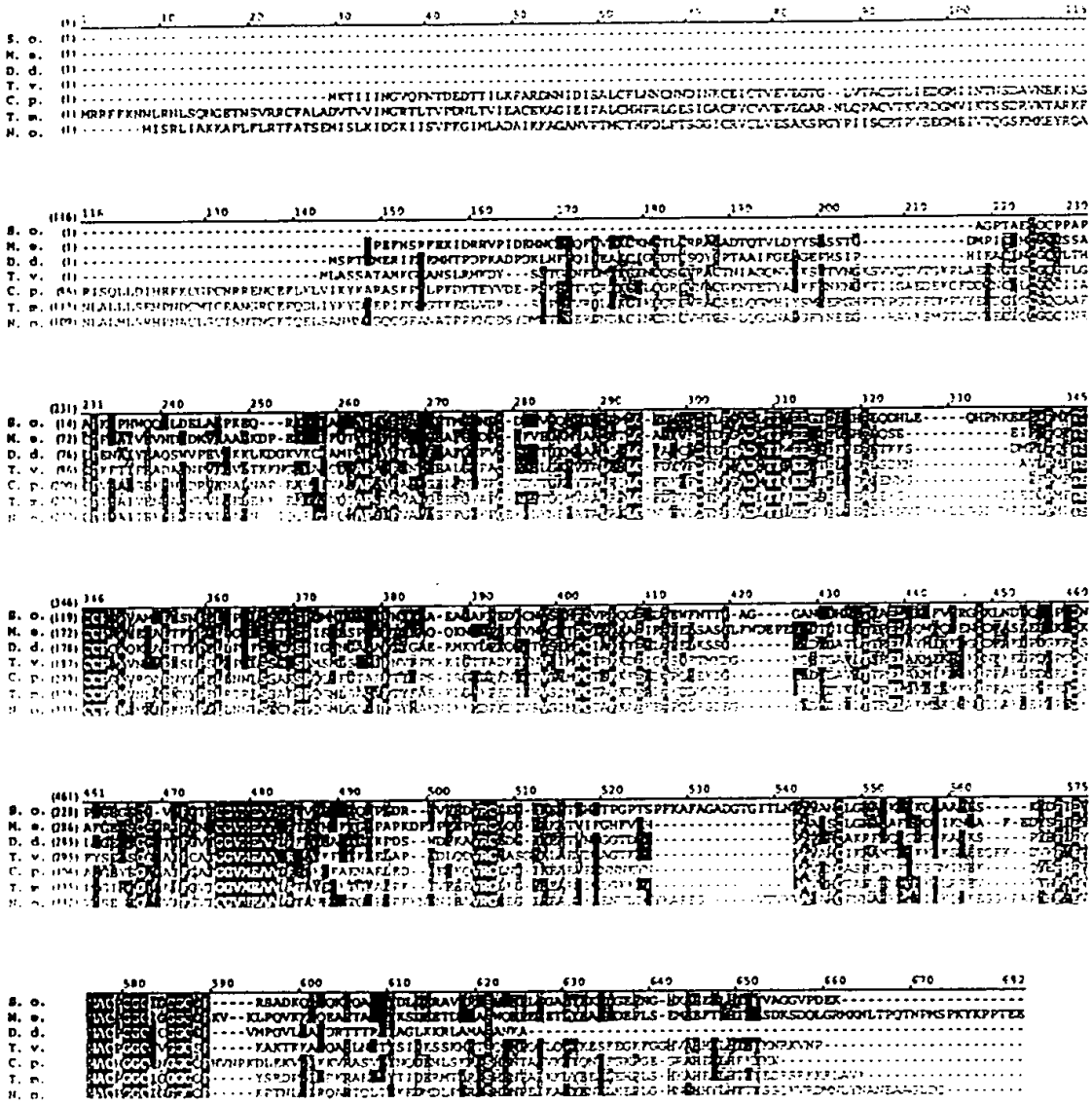

FIG. 3. Comparison of the deduced HydA protein sequence with other iron hydrogenases. The protein alignment was done by using the Vector NTI program (InforMax). *White letters* with *black background* indicate amino acids identical to the HydA protein. *Black letters* with *gray background* indicate conserved changes of the amino acids. *S. o.*, *S. obliquus* (this work); *M. e.*, *Megasphaera elsdenii* (13); *D. d.*, *D. desulfuricans* (17); *T. v.*, *Trichomonas vaginalis* (10); *C. p.*, *C. pasteurianum* (9); *T. m.*, *T. maritima* (12); *N. o.*, *N. ovalis* (11).

derived from conserved regions within the *C. reinhardtii hydA* gene.[2] The complete cDNA clone of 2609 bp was obtained by 5'- and 3'-RACE PCR. It contains an open reading frame of 1344 bp encoding a polypeptide of 448 amino acids (Fig. 1) followed by an extensive 3'-untranslated region of about 1100 bp. The coding region of *S. obliquus hydA* exhibits features common to other green algae such as high GC content (64.2%) and a characteristic putative polyadenylation signal, TGTAA, 15 bp upstream of the poly(A)$^+$ sequence (45).

In an effort to examine the exon-intron structure and the promoter region of the *hydA* gene, about 5 kilobases of the genomic DNA from *S. obliquus* were sequenced. The gene comprises five introns with a total size of 1310 bp (Fig. 1) whose 5' and 3' ends contain typical plant splice donor and acceptor sites that follow the GT/AG rule.

A genomic Southern blot was probed with a 750-bp PCR fragment to determine the copy number of the *hydA* gene (Fig. 2). Single bands were observed in lanes with samples digested with *Hin*cII, *Eco*RV, and *Nde*I and a double band in the lane containing genomic DNA digested with *Sac*I. The band migration positions matched the sizes predicted from the sequence of the *hydA* gene, indicating that HydA is encoded by a single copy gene (Fig. 2). The same hybridization pattern was observed even under low stringency conditions (hybridization temperature 50 °C; data not shown). The transcription start position was determined by primer extension using RACE-PCR and was found 139 bp upstream of the ATG start codon. We designed several primers within 100 bp of the 5' end of the known *hydA* cDNA to confirm the accuracy of the transcription initiation site. All of the sequenced PCR clones had the same 5' ends at position +1. As described for other green algae genes, a highly conserved TATA box element upstream of the transcription starting point is absent (46). However, the TACATAT motive at position −25 in a GC-rich region shows similarities to other TATA motives in *C. reinhardtii* and therefore might be involved in gene expression.

HydA Is a Novel Type of Iron Hydrogenase—The polypeptide derived from the cDNA sequence has a length of 448 amino

[2] T. Happe, unpublished results.

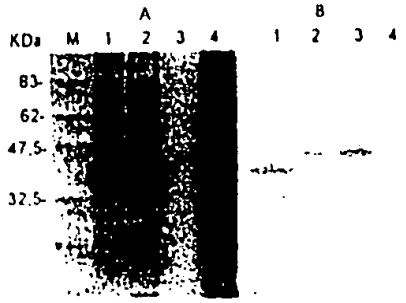

FIG. 4. **Recombinant expressed HydA reacts with antibodies raised against *C. reinhardtii* iron hydrogenase.** The *hydA* gene coding region corresponding to amino acid 36 to residue 448 was cloned *Nde*I-*Bam*HI into pET9a. HydA protein was expressed upon induction with IPTG. *Lane 1*, recombinant expressed HydA protein from *S. obliquus*; *lane 2*, recombinant expressed iron hydrogenase from *C. reinhardtii*; *lane 3*, purified hydrogenase from *C. reinhardtii*; *lane 4*, total proteins from induced *E. coli* cells without plasmid. *A*, SDS-PAGE. *Lane M*, molecular mass marker (Bio-Rad) indicating relative molecular masses in kDa. SDS-PAGE stained with Coomassie Blue is shown. *B*, Western blot probed with HydA antibody. The recombinant proteins of *lanes 1* and *2* from A were diluted 1:10.

acids and a predicted molecular mass of 48.5 kDa (44.5 kDa without transit peptide, respectively); consequently, HydA is the smallest hydrogenase protein known so far. The N terminus of HydA is basic and contains numerous hydroxylated amino acids and an Val-Xaa-Ala motive at position 35, a characteristic feature of chloroplast transit peptides (47, 48).

The processed HydA protein is compared with four bacterial and two eukaryotic iron hydrogenases as shown in Fig. 3. The homology in the C-terminal region of all proteins is quite striking. For example, the *S. obliquus* HydA protein shows 44% identity and 57% similarity to the *C. pasteurianum* iron hydrogenase (9). The H cluster in *S. obliquus* might be coordinated by four cysteine residues at positions 120, 175, 335, and 340. Other strictly conserved amino acid structures like FTSCCPGW (343–350), TGGVMEAALR (474–483), and MACPGGCXXGGGQP (576–589) probably define a pocket surrounding the active center as shown by the structural data of *C. pasteurianum* and *D. desulfuricans* (16, 17). On the other hand, the N-terminal region is completely different from all other iron hydrogenases. The protein sequences of the other enzymes comprise at least two [4Fe-4S] ferredoxin-like domains (called F cluster) that are necessary for the electron transport from the electron donor to the catalytic center. The iron hydrogenases of *C. pasteurianum*, *Thermotoga maritima*, and *Nyctotherus ovalis* (9, 12, 11) contain an extra [4Fe-4S] cluster and one [2Fe-2S] center. This N-terminal domain with the F cluster or other [Fe-S] centers is completely lacking in HydA of *S. obliquus*. This indicates that there is a direct electron transport pathway from the exogenous donor to the H cluster.

To verify that the isolated cDNA encodes a iron hydrogenase, the *hydA* clone was expressed in the heterologous system *E. coli*. One band of recombinant HydA protein was observed on SDS-PAGE at ~44 kDa, in agreement with the molecular mass of the polypeptide predicted from the cDNA sequence. Antibodies raised against the HydA protein of *C. reinhardtii*, which cross-react with other iron hydrogenases but not with nickel-iron hydrogenases (data not shown), were applied in Western blot analysis. One distinct signal with the overexpressed HydA protein of *S. obliquus* was obtained (Fig. 4).

The lysate of induced *E. coli* cells exhibited no hydrogenase activity. This result corresponds to observations by Voordouw *et al.* (50) and Stokkermans *et al.* (51), who also detected no H_2 production of recombinant iron hydrogenases in *E. coli* cells. The reason for that might be that the bacterial cells do not have the ability to assemble the special H cluster of iron hydrogenases.

Rapid Induction of hydA mRNA during Anaerobic Adaptation—The regulation of the *hydA* gene expression was examined by Northern blot analysis and reverse transcription-PCR. Aerobically grown cells of *S. obliquus* did not show a hydrogenase activity (Fig. 5A). Total RNA and also mRNA were isolated from cells that were induced by argon bubbling for 0, 1, and 4 h. Northern blot analysis and reverse transcription-PCR demonstrated that the *hydA* gene is expressed after anaerobic adaptation. There is a very weak signal without adaptation ($t = 0$), but strong signals of the transcript could be detected after anaerobic induction (Fig. 5, *B* and *C*). The full length of the *hydA* cDNA clone was confirmed by the transcript signal (2.6 kilobases) on the Northern blot.

DISCUSSION

In green algae, the occurrence of a hydrogen metabolism induced by anaerobic conditions is well established. Despite the great interest in hydrogen evolution for practical applications ("biophotolysis"), the hydrogenase genes from green algae have not yet been isolated. The *hydA* gene and the isolated HydA protein of *S. obliquus* that we present in this work belong to the class of iron hydrogenases.

Iron hydrogenases have been isolated only from certain anaerobic bacteria and some anaerobic eukaryotes as well as from the anaerobically adapted green alga *C. reinhardtii* (30). The enzymes are found to exist in monomeric (9, 13, 53, 54), dimeric (17), and multimeric (12) forms; however, in eukaryotes only monomeric proteins have been isolated (10, 11).

The HydA protein of *S. obliquus* is synthesized in the cytoplasm. The first 35 residues (Met^1–Ala^{35}) of the amino acid sequence derived from the cDNA sequence are supposed to function as a short transit peptide that routes the nuclear encoded protein to the chloroplast. Several positively charged amino acids that describe a typical feature for algal transit peptides (47) are found in HydA. The three terminal residues of the signal sequence, Val-Xaa-Ala, constitute the consensus sequence for stromal peptidases (48).

The hydrogenase of *S. obliquus* represents a novel type of iron hydrogenase. The monomeric enzyme of 448 amino acids and a calculated molecular mass of 44.5 kDa for the processed protein is the smallest iron hydrogenase isolated so far. The protein sequence consists of an unusual N-terminal domain and a large C-terminal domain containing the catalytic site. The structurally important C terminus of the *S. obliquus* HydA sequence is very similar to that of other iron hydrogenases. Four cysteine residues at positions 120, 175, 336, and 340 coordinate the special [6Fe] cluster (H cluster) of the active site (Fig. 6). A number of additional residues define the environment of the catalytic center. Peters *et al.* (16) postulated 12 amino acids in *C. pasteurianum* to form a hydrophobic pocket around the cofactor. Ten residues are strictly conserved, while two amino acids vary within the iron hydrogenase family (Ser^{232} and Ile^{268} in *C. pasteurianum*, Ala^{119} and Thr^{155} in *T. vaginalis*, and Ala^{44} and Thr^{60} in *S. obliquus*). A small insertion of 16 amino acids is noted in *S. obliquus*, but this addition occurs in an external loop of the protein and probably has no special function (Fig. 6).

Until now, all iron hydrogenases possessed a ferredoxin-like domain in the N terminus coordinating two [4Fe4S] clusters (FS4A and FS4B; Refs. 10 and 13 and Fig. 6). The iron sulfur clusters facilitate the transfer of electrons between external electron donors or acceptors and the H cluster. The N terminus of the *S. obliquus* protein is strongly reduced compared with other iron hydrogenases, and no conserved cysteines are found. Therefore, we postulate that all accessory Fe-S clusters (FS2,

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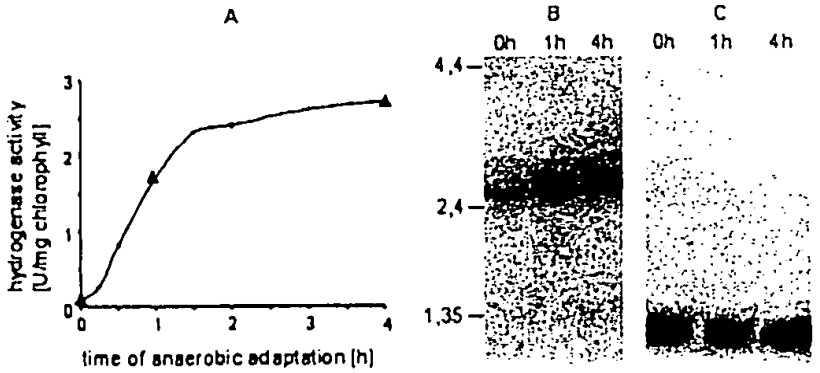

FIG. 5. **Induction of the hydrogenase activity and differential expression of the *hydA* gene during anaerobic adaptation.** *A*, *S. obliquus* cells were anaerobically adapted by flushing the culture with argon in the dark. After removing cell samples at the indicated times, the algae were broken by Triton X-100 treatment. The *in vitro* hydrogenase activity was measured as described under "Experimental Procedures." *B*, Northern hybridization was performed with the *hydA*-specific probe. Adapted cells were harvested at 0, 1, and 4 h, and the RNA was isolated 20 μg of total RNA was loaded per lane. *C*, the same RNA was hybridized with a constitutive expressed gene (plastocyanin) as control.

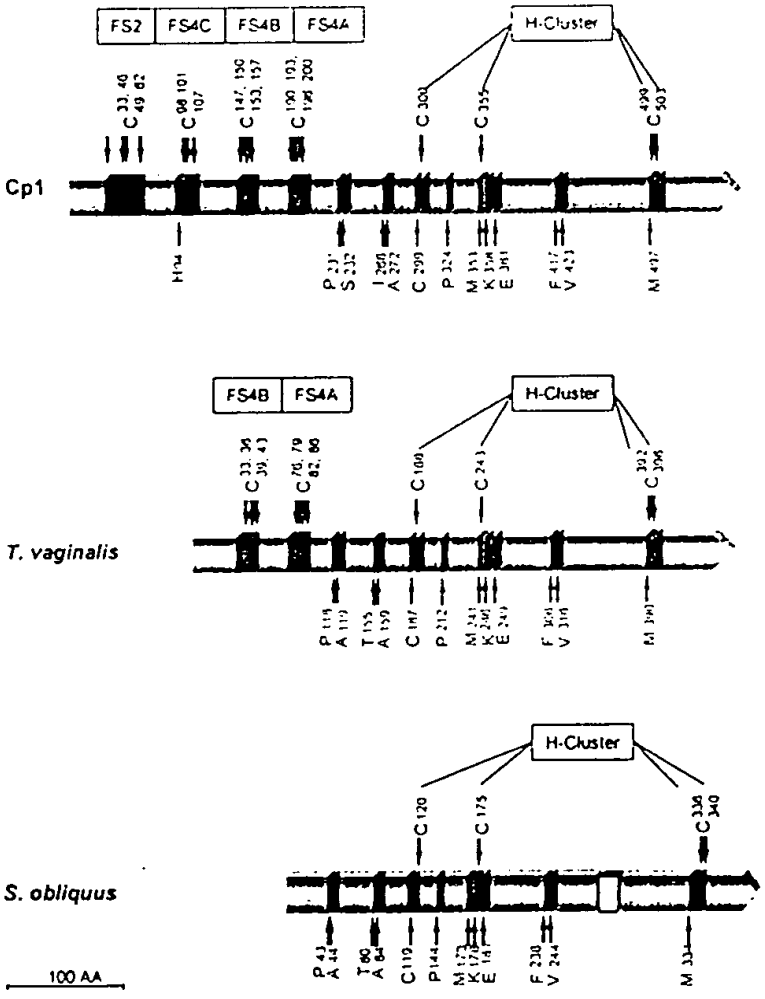

FIG. 6. Schematic alignment of the conserved cysteine residues and other important amino acids of the H cluster. The protein is illustrated as a *large gray arrow*. *Small arrows* indicate parallelograms which demonstrate conserved amino acids in the protein. Cysteines participating at the coordination of the [Fe-S] clusters are *gray*, whereas identical amino acids are *black*. An insertion of 16 amino acids in the *S. obliquus* protein is illustrated as a *spotted bar*. *FS4*, [4Fe-4S] cluster; *FS2*, [2Fe-2S] cluster.

FS4A, FS4B, and FS4C) are missing. No hints for a second subunit have been observed during purification of the protein.

In contrast to earlier observations in *S. obliquus* (32), we could neither detect the postulated two subunits of a potential nickel-iron hydrogenase nor find a nickel dependence related to the hydrogenase activity. Francis reported about two forms of hydrogenases in *S. obliquus* (52), but although we used the same alga strain and identical adaptation conditions, we were not able to detect a second hydrogenase activity during the purification steps.

Physiological studies have shown that the hydrogen evolution is coupled to the light reaction of the photosynthesis (24–26). In contrast to earlier observations in *S. obliquus* (25, 26), we measured PSII independent H_2 production that is not influenced by 3-(3,4-dichlorophenyl)-1,1-dimethylurea. The electrons required for H_2 evolution come from redox equivalents of the fermentative metabolism and are supplied into the photosynthetic electron transport chain via the plastochinone pool.

For the first time we demonstrate that the ferredoxin PetF functions as the *in vivo* electron donor of the iron hydrogenase

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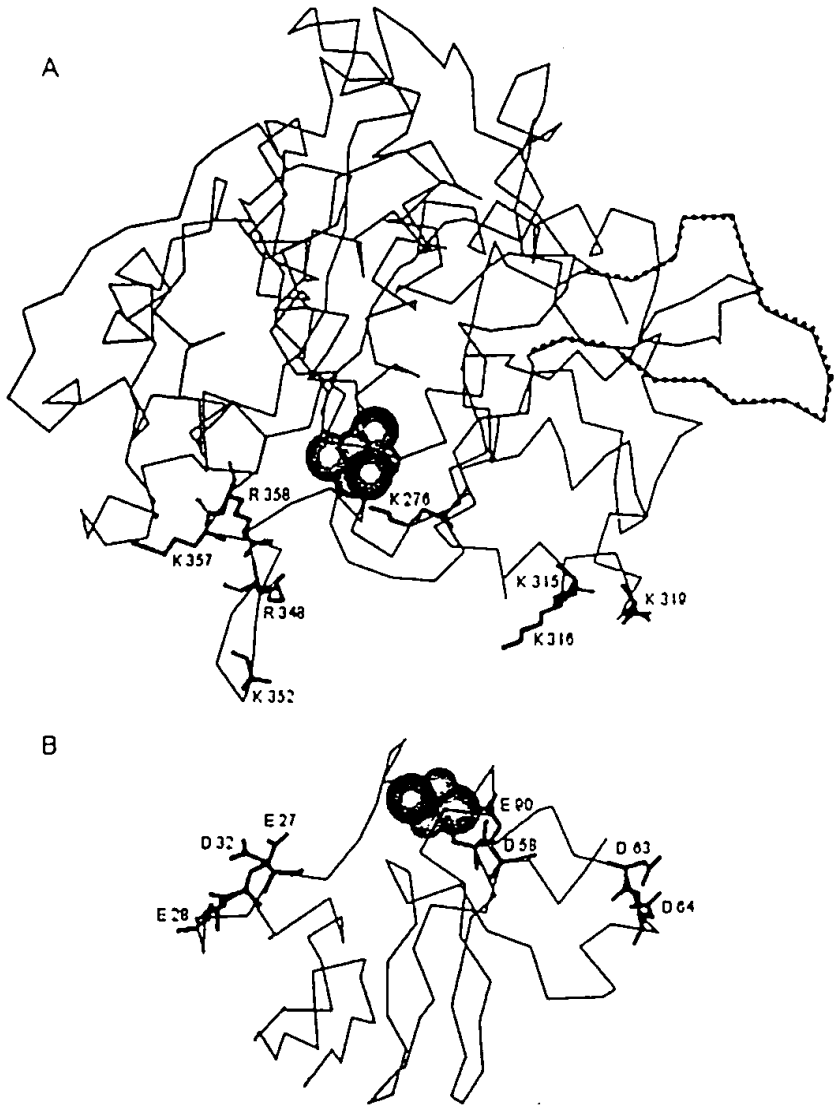

FIG. 7. **Schematic view of the structures of *S. obliquus* HydA (*A*) and the electron donor ferredoxin (*B*).** The figure shows the α carbons and the side chains of charged residues that might be important for the electron transfer reaction or the interaction between HydA and the ferredoxin from *S. vacuolatus* (58). The 16-amino acid insertion of the hydrogenase appears as external loop and is distinguished as *dotted line*. The amino acid sequence of the mature HydA protein (His^{19}–Tyr^{404}) was submitted to the SWISS-MODEL server (59). We generated a model of HydA with the known three-dimensional structure of the iron hydrogenase from *C. pasteurianum* (16) as template, sharing 57% sequence identity with the submitted sequence. The Protein Data Bank file was visualized by the Swiss-PDB viewer (57).

from *S. obliquus*. Hydrogenase activity can be specifically blocked by addition of the ferredoxin antagonist sulfo-disalicylidinepropandiamine (55) and antibodies raised against the PetF protein. *In vitro*, a hydrogen evolution by HydA was only measured with plant-type [2Fe-2S] ferredoxins like PetF of *S. obliquus*, *C. reinhardtii*, and spinach as electron mediators. Bacterial iron hydrogenases are known to be reduced by [4Fe-4S] ferredoxins and do not accept electrons from plant-type proteins (56).

The analysis of the three-dimensional structure of the iron hydrogenase from *C. pasteurianum* (CpI) gave evidence that the interaction with external electron donors might occur at the accessory [Fe-S] clusters in the N-terminal domain (14). Based on the x-ray structure of CpI, we modeled the iron hydrogenase of *S. obliquus* (57). As shown in Fig. 7, a region of positive surface potential is observed within HydA based on a local concentration of basic residues. In contrast to the docking position of ferredoxin in CpI, these charged amino acids in the *S. obliquus* iron hydrogenase are located within the C-terminal domain, forming a niche for electron donor fixation.

The known algal ferredoxin proteins exhibit high degrees of sequence identity (over 85%), and the charged amino acids are strictly conserved. The petF sequence of *S. obliquus* is unknown, but very recently the x-ray model of the ferredoxin from another *Scenedesmus* species (*Scenedesmus vacuolatus*; Ref. 58) was published. The structure revealed negatively charged amino acids like aspartate and glutamate near the [2Fe-2S] cluster. The [Fe-S] center and the H cluster of the hydrogenase probably come into close proximity through electrostatic interactions. This geometry is consistent with efficient electron transfer among these prosthetic groups.

As already shown in various studies, a correlation exists between the duration of time of the anaerobic adaptation and the increase of hydrogen production (30, 32). Reverse transcription-PCR and Northern blot analyses with mRNA of aerobic and anaerobically adapted cells from *S. obliquus* showed an increased level of *hydA* transcript after 1 h of induction. Correspondingly, hydrogen evolution was only measured after a short time of anaerobic adaptation. These results suggest that the expression of the *hydA* gene is regulated at the transcriptional level. The small amount of transcript that was detected at $t = 0$ may be due to transcript synthesis induced by microanaerobic conditions during the RNA isolation procedure. Alternatively, a low level of *hydA* transcript might be constitutively present in the cell and is only drastically increased after anaerobic adaptation.

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AQ: L

AQ: M

AQ: N

COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No.: 01MEL1 PATENT

As a below-named inventor, I HEREBY DECLARE THAT:
This Declaration is for the following type application: ☒ ORIGINAL; ☐ DESIGN; ☐ CONTINUATION; ☐ DIVISIONAL; ☐ CIP; ☐ National Stage of PCT; ☐ SUPPLEMENTAL.
(see attached two pages if SUPPLEMENTAL is indicated)

My residence, post office address and citizenship are as stated below next to my name; I believe that I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled : **Hydrogen Production**, the specification of which (check one) : ☒ **is attached hereto**; ___ was filed on _____ as Application Serial No. _____ and was amended on (or amended through _____, if applicable); ___ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____ (if any).

I hereby state that I have reviewed and understand the contents of the specification, claims, and any amendment(s) referred to above. I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations § 1.56 (a).

☐ In compliance with this duty there is attached an Information Disclosure Statement 37CFR1.97 (37CFR156 (a)).

I hereby claim Foreign priority benefits under Title 35, united States Code § 119 of any foreign applications(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) designating at least one country other than the United States of America or filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

☒ **No such applications have been filed;** ☐ such applications have been filed as follows (see attached page)

Prior Foreign Application(s)		Month/Day Year Filed	Priority Claimed Under 35 USC 119	
Country: _____	Number: _____	_____	Yes _____	No _____
Country: _____	Number: _____	_____	Yes _____	No _____
Country: _____	Number: _____	_____	Yes _____	No _____

POWER OF ATTORNEY

I hereby appoint Michael G. Petit, Registration No. 30, 795, as my attorney with full powers of substitution and revocation, to prosecute his application and transact all business in the Patent and Trademark Office connected herewith.

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DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Applicant or Patentee: Thomas Happe Attorneys Docket No.: 01MEL1
Serial or Patent No.: Not Available
Filed or Issued: Concurrently Herewith
For: Hydrogen Production.

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(F) AND 1.27(B) - INDEPENDENT INVENTOR

As a below named inventor, I hereby declare that I qualify as an independent inventor as defined in 37 CFR 1.9(c) for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, to the Patent and Trademark Office with regard to the invention entitled: **Hydrogen Production**, described in

- ☒ the specification filed herewith.
- ☐ application serial no. _____, filed _____.
- ☐ patent no. _____ issued _____.

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I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (36 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Thomas Happe
NAME OF INVENTOR

Thomas Happe
SIGNATURE OF INVENTOR

January 24, 2001
DATE